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Immunogen-dependent quantitative and qualitative differences in phagocytic responses of the circulating hemocytes of the lobster *Homarus americanus*

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ABSTRACT: Phagocytic responses in circulating hemocytes of the lobster *Homarus americanus* were measured before and after treatment of lobsters with 2 different immunogens: (1) lipolysaccharide (LPS) or endotoxin from a non-pathogenic *Pseudomonas perolens*, and (2) a vancomycin/live Gram-positive pathogen (*Aerococcus viridans* [var.] *homari*) combination, essentially attenuated cells, shown previously to induce a high degree of resistance to this pathogen. The responses elicited by each of the immunogens were markedly different. Hemocytes drawn from LPS-treated lobsters showed significant, largely non-specific, increases in phagocytic responses over baseline values against sheep red blood cells and an array of test bacteria, with the notable exception of the pathogen. In marked contrast, induction with the vancomycin/live pathogen combination resulted in highly significant and specific increases in phagocytic responses to the pathogen and to the related, (but avirulent) strains of the pathogen, as well as inducing in the lobsters the usual high degree of resistance to the pathogen. These results suggest that quantitative and qualitative variations in phagocytic and resistance levels induced in at least 1 crustacean genus are determined largely by the particular characteristics of the immunogen.

KEY WORDS: Immunogens · Phagocytosis · Hemocytes · Lobsters · *Homarus americanus* · *Aerococcus viridans* (var.) *homari*

INTRODUCTION

Gaffkemia, the fatal systemic infection of homarid lobsters caused by the free-living, Gram-positive, tetrad-forming coccus *Aerococcus viridans* (var.) *homari* (formerly *Gaffkya homari*), causes substantial economic losses. Periodically, both *Homarus americanus* and *H. gammarus* experience epizootics of gaffkemia in the wild and are subject to major outbreaks in the communal units where the live lobsters are held for days to months awaiting sale and delivery to the consumers. Reviews of the disease include those by Stewart & Rabin (1970), Fisher et al. (1978), Sparks (1981), Stewart (1984, 1993) and Sindermann (1990).

The pathogen lacks invasive powers, and although lobsters are often cannibalistic the pathogen is not transmitted orally, as the lobster gastric fluid is lethal toward it. Infection of lobsters occurs only when their highly effective integument is breached in the presence of a virulent strain of the pathogen; virulent strains have proven to be resistant to all internal lobster defenses (Stewart et al. 1969b). Virulence is related to possession of a pronounced polysaccharide capsule; avirulent strains exhibit none or only traces of a capsule. Virulent strains of *Aerococcus viridans* (var.) *homari* tend to lose their capsules and their virulence gradually with prolonged culture in the standard laboratory medium trypticase soy broth; the capsule

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and accompanying virulence have been restored in avirulent strains by growing them in lobster hemolymph serum (Stewart et al. 2004b). Bacterins prepared using this pathogen have induced in lobsters different degrees of resistance which appear to vary as a function of the treatment applied to the immunogen(s) (Stewart & Zwicker 1974, Rittenburg 1981, Keith et al. 1992, Stewart et al. 2004a). Some of these treatments clearly involve alterations of the capsule and increased cellular permeability of the pathogen (Stewart et al. 2004a).

As summarized by Johansson et al. (2000), the circulating hemocytes of crustaceans and other invertebrates play a central role in the diverse and complex defense system, where they perform essential functions such as phagocytosis, encapsulation and lysis of foreign cells. Although phagocytosis was shown by Cornick & Stewart (1968) to occur *in vivo* early in gaffkemia, it failed to halt the infection. In fact, the hemocytes disappeared and the released bacterium *Aerococcus viridans* (var.) *homari* flourished in the various tissues, eventually leading to the death of the host (Stewart et al. 1969a, Johnson et al. 1981, Stewart et al. 1983). Phagocytosis in lobsters, but not resistance to *A. viridans* (var.) *homari*, was enhanced substantially by injection of the Gram-negative bacterium *Pseudomonas perolens* (non-pathogenic to lobsters) and its endotoxin (Paterson & Stewart 1974). Phagocytosis was also enhanced slightly by formolized *A. viridans* (var.) *homari* (Paterson et al. 1976). The trials indicated that different levels of phagocytosis occurred as a function of the immunogen as well as the test particles (erythrocytes or bacteria) used to measure the response. In addition, in limited trials, Rittenburg (1981) and Stewart et al. (2004a) showed that 2 quite differently prepared bacterins of *A. viridans* (var.) *homari* induced resistance to the pathogen coincident with minor increases in phagocytosis of the pathogen.

The present study was carried out to determine, through direct comparison, the degree to which the phagocytic responses induced by 2 quite different immunogens *Pseudomonas perolens* lipolysaccharide (LPS) or endotoxin and a vancomycin/live pathogen-attenuated cell (*Aerococcus viridans* [var.] *homari*) combination (Stewart & Zwicker 1974) differed quantitatively and qualitatively.

MATERIALS AND METHODS

Lobsters. Freshly captured *Homarus americanus* (450 to 500 g each) were obtained directly from local fishermen. Their large chelae were immobilized with heavy rubber bands and they were acclimated to 15°C for 10 d in tanks supplied with continuously flowing, filtered, aerated seawater. Throughout the experimental series, they were fed ad libitum twice a week with beef liver alternating with sardine herring. Each tank contained sufficient individual shelters to house all lobsters; a separate tank was used for each treatment and for each control.

Bacteria. The virulent strain of *Aerococcus viridans* (var.) *homari* used throughout this study was donated by Dr. H. Rabin, Department of Pathobiology, The Johns Hopkins University, Baltimore, Maryland, USA. Its virulence was maintained by serial passage through lobsters held at 15°C (Cornick & Stewart 1968). The 2 avirulent strains of *A. viridans* (var.) *homari* were (1) American Type Culture Collection (ATCC) 10400, and (2) 37R, a strain isolated during a field survey (Stewart et al. 1966); both were shown to be avirulent by challenges of live lobsters. Confirmation that all these *A. viridans* (var.) *homari* are taxonomically correctly placed has been provided recently by Greenwood et al. (2005). The *Pseudomonas perolens* strain was isolated from the intestine of a lobster by Stewart & Zwicker (1972), who showed that it was non-pathogenic for *Homarus americanus* and that formalin-killed preparations of the strain did not protect lobsters against infection by *A. viridans* (var.) *homari*. For use as test particles in measuring phagocytosis, all bacteria were grown for 24 h in trypticase soy broth at 28°C.

Immunogens and immunostimulation. (1) Lipopolysaccharide or endotoxin extracted from *Pseudomonas perolens* by the method of Staub (1967) was treated with formalin and made up to a concentration of 200 µg ml^{-1} LPS in 3% NaCl; this immunogen was injected at the rate of 1.0 ml kg^{-1} host body wt (BW). (2) Following the procedure of Stewart & Zwicker (1974), lobsters were injected with 1.0 mg kg^{-1} BW of vancomycin (vancomycin hydrochloride; Eli Lilly & Co.) in 3% NaCl, followed 24 h later by injection of 1.0 ml kg^{-1} BW of a suspension (6.2×10^5 cells ml^{-1}) of 24 h live cells of *Aerococcus viridans* (var.) *homari* (Rabin's strain). All injections were made in the ventral abdominal sinus; the site was swabbed with 70% ethanol before and after injection.

Sterile hemolymph serum. Based on the procedure of Lauffer & Swaby (1955), hemolymph drawn from the ventral abdominal sinus was allowed to form a non-retracting, firm, hard clot. The clot was broken up and centrifuged at $12\,800 \times g$ for 10 min; the supernatant fluid was passed through coarse filter-paper at 5°C, then sterilized by membrane filtration (0.45 µm pore-size) and stored at 4°C. The serum used had an agglutinin titer of 128 or 256 (reciprocals of the dilutions) tested against 1% suspensions of sheep erythrocytes. The lobster hemolymph sera were used to opsonize the 10% bacterial and sheep red blood cell (SRBC) suspensions for 1 h at 20°C as described by Paterson & Stewart (1974).

Phagocytosis. Phagocytosis of bacteria and SRBC hemocytes was determined at 15°C using the protocols described by Paterson & Stewart (1974) and Paterson et al. (1976). The glucose concentration of the lobster hemolymph medium (LHM) was doubled to 1.0 g l^{-1} as this enhanced hemocyte viability. The percentages of hemocytes showing phagocytosis and the number of particles of bacteria or erythrocytes contained in each hemocyte were determined using phase-contrast microscopy at a magnification of 790× to examine between 250 and 500 intact hemotcytes per reported value.

Blockage of phagocytic effects by mucin and *Aerococcus viridans* (var.) *homari* capsular material. The major glycoprotein from bovine submaxillary mucin (Worthington Biochemicals) was purified according to the procedures of Gottschalk & Bhargava (1972). The capsular material from the pathogen was prepared as described by Kenne et al. (1976), with chemical analyses of capsular material provided by Hermansson et al. (1990). Each of these additives was dissolved in LHM at concentrations of 2 mg ml^{-1}, and the pH was adjusted to 7.6.

For determination of effects, 2 ml of each solution was mixed with 2 ml of opsonized bacteria (40% suspension in LHM) resulting in a final 20% bacterial suspension. After incubation of this mixture at 20°C for 1 h, 0.1 ml of these suspensions was added to each appropriate Leighton tube immediately after addition of the hemocytes, for the standard measurement of phagocytosis.

Statistical analysis. All statistical analyses were performed using SPSS®, 12.0. For percentages of hemocytes showing phagocytosis, a Kruskal-Wallis *H*-test followed by a Mann-Whitney *U*-test were carried out on the raw data (see Tables 1 & 3), a GLM (generalized linear model) ANOVA with Tukey's HSD-test was used to identify significant differences between treatments (see Tables 4 to 6), and between each treatment the data was arcsine-transformed prior to analysis using independent 2-sample *t*-tests to identify significant differences (see Tables 1, 3 to 6). To determine whether significant differences existed in the number of particles per hemocyte showing phagocytosis (see Tables 4 & 6), the Kruskal-Wallis *H*-test followed by a Mann-Whitney *U*-test was employed (for comparisons of Strain 10400 the Mann-Whitney *U*-test was performed on the raw data).

RESULTS

The baseline phagocytic values for hemocytes from untreated lobsters (Table 1) varied considerably, depending on the test particles used. The same variation was not evident in the number of particles phagocytosed per hemocyte, as these values remained relatively constant. It is noteworthy that phagocytosis of the virulent strain of the pathogen was extremely small compared with that of the other particles.

As illustrated in Table 2, all treatments administered to the lobsters gave results comparable to those recorded previously in similar trials. Lobsters injected with the vancomycin/live suspension of virulent *Aerococcus viridans* (var.) *homari* cell combination exhibited a high degree of resistance to the pathogen 27 d later. In contrast, the group of lobsters treated with vancomycin alone suffered 100% mortalities when challenged 27 d later, when the antibiotic had been eliminated. Untreated lobsters were completely susceptible to the pathogen.

With 1 notable exception, the phagocytic values for the hemocytes from lobsters given *Pseudomonas perolens* LPS exhibited substantial and significant increases (Table 3). The sole exception was test particles of virulent *Aerococcus viridans* (var.) *homari*, where no significant increase occurred. In contrast, the phagocytic capacities of hemocytes from lobsters treated with the vancomycin/live *A. viridans* (var.) *homari* (Rabin's) combination increased to highly significant levels against all strains of the pathogen, virulent as well as avirulent (Table 4). In addition to the increases in the percentages of hemocytes exhibiting phagocytosis, this same group of hemocytes also showed significant increases in the number of particles per hemocyte displaying phagocytosis. A significant increase in phagocytic values was observed for the hemocytes tested against *P. perolens*,

Table 1. *Homarus americanus.* Baseline phagocytic capacities (means ± SD calculated from individual determinations for each lobster) of hemocytes from untreated lobsters; different superscripts denote significant differences ($p < 0.05$). Data without superscripts are not significantly diffferent. n: no. of lobsters

Opsonized test particles	n	% hemocytes showing phagocytyosis	No. particles hemocyte^{-1} showing phagocytosis
Aerococcus viridans (var.) *homari*			
Virulent strain:			
Rabin's	5	0.8 ± 0.7[a]	2.3 ± 0.4
Avirulent strains:			
ATCC 10400	5	6.2 ± 2.8[b]	2.7 ± 1.2
37R	5	5.2 ± 2.3[b]	2.8 ± 1.6
Pseudomonas perolens	5	10.4 ± 5.1[b]	4.2 ± 3.5
Sheep red blood cells	5	4.4 ± 1.5[b]	2.1 ± 1.3

but not for SRBC (Table 4). This increase was less than that observed for hemocytes taken from lobsters treated with LPS (cf. Table 3); apparently this increase was part of a generalized, non-specific response.

Tables 5 & 6 illustrate that pronounced and significant effects of the 2 additives (mucin or capsular material) occurred only when they were incorporated into the system containing the avirulent ATCC 10400 test particles with hemocytes drawn from lobsters immunized with the vancomycin/live pathogen combination (Table 6). The significant reduction in the percentage of hemocytes showing phagocytosis is further evidence of a specific qualitative change in responses induced by the antibiotic/live pathogen combination.

In general, the injection of *Pseudomonas perolens* LPS induced non-specific increases over baseline phagocytic values that were approximately double those induced by the avirulent *Aerococcus* strains and 3 times higher than those induced by *P. perolens* and SRBC (Table 3). In contrast, the vancomycin/live pathogen combination produced only a doubling of the phagocytic baseline values for *P. perolens* and SRBC, a 4- to 5-fold increase in the (already appreciable) phagocytosis values for the avirulent strains, but a major and highly specific increase in phagocytosis of the virulent *Aerococcus* strain *viridans* (var.) *homari*, i.e. 18.25-fold or a 1725% increase (Table 4).

Table 2. *Homarus americanus.* Impact of various treatments on lobsters. BW: body weight; n: no. of lobsters tested; Survival after 27 d: no. surviving 27 d after treatment; Challenge after 27 d: no. challenged with *A. viridans* (var.) *homari* (Rabin's) 27 d after treatment, challenge dose = 7.4×10^5 cells kg^{-1} BW: Survival after 21 d: no. of infection-free survivors 21 d after challenge; Mortality: no. of deaths from gaffkemia

Treatment	n	Survival after 27 d	Challenge after 27 d	Survival after 21 d	Mortality
Pseudomonas perolens endotoxin (200 µg kg^{-1} BW)	20	20	0	20	0
Vancomycin · HCl (1 mg kg^{-1}) followed by live *Aerococcus viridans* (var.) *homari* Rabin's 6.2×10^5 cells kg^{-1} BW) 24 h later	20	20	10	7	2
3% NaCl (1 ml kg^{-1} BW)	5	5	0	5	0
Vancomycin · HCl (1 mg kg^{-1} BW)	5	5	5	0	5
Live pathogen (Rabin's) (6.2×10^5 cells kg^{-1} BW)	5	0	–	–	5

Table 3. *Homarus americanus.* Phagocytic capacities (means ± SD calculated from individual determinations for each lobster) of hemocytes from lobsters injected with *Pseudomonas perolens* endotoxin 27 to 35 d earlier. Increase in phagocytosis: multiples of increases over baseline values in Table 1. Different superscripts denote significant differences among treatments in column ($p < 0.05$); *: significant difference from corresponding value in Table 1 ($p < 0.05$); n: no. of lobsters

Opsonized test particles	n	% hemocytes showing phagocytyosis	Increases in phagocytosis	No. of particles hemocyte^{-1} showing phagocytosis
Aerococcus viridans (var.) *homari*				
Virulent strain: Rabin's	5	1.2 ± 0.7^{a}	1.5	2.6 ± 1.0
Avirulent strains: ATCC 10400	5	$13.0 \pm 2.4^{b,*}$	2.1	2.9 ± 1.4
37R	5	$10.6 \pm 4.0^{b,*}$	2.0	3.3 ± 2.1
Pseudomonas perolens	5	$31.6 \pm 6.2^{c,*}$	3.0	3.9 ± 3.7
Sheep red blood cells	5	$12.4 \pm 3.6^{b,*}$	2.8	2.7 ± 1.9

Table 4. *Homarus americanus.* Phagocytic capacities of hemocytes from lobsters injected 27 to 35 d earlier with vancomycin followed 24 h later with live *Aerococcus viridans* (var.) *homari* (Rabin's). Further details in Table 3

Opsonized test particles	n	% hemocytes showing phagocytyosis	Increases in phagocytosis	No. of particles hemocyte^{-1} showing phagocytosis
Aerococcus viridans (var.) *homari*				
Virulent strain: Rabin's	5	$14.6 \pm 3.9^{ab,*}$	18.25	6.4 ± 6.1^{a}
Avirulent strains: ATCC 10400	5	$23.6 \pm 6.0^{ab,*}$	3.8	5.6 ± 5.2^{a}
37R	5	$26.4 \pm 12.1^{b,*}$	5.2	6.0 ± 5.8^{a}
Pseudomonas perolens	5	$23.2 \pm 9.5^{ab,*}$	2.23	3.7 ± 2.9^{b}
Sheep red blood cells	5	9.2 ± 4.7^{a}	2.1	3.0 ± 2.1^{b}

Table 5. *Homarus americanus*. Effects of mucin and *Aerococcus viridans* (var.) *homari* capsular material on phagocytic capacities of hemocytes from lobsters injected with *Pseudomonas perolens* endotoxin 37 to 42 d earlier. Further details as in Table 3

Opsonized test particles (*Pseudomonas perolens*)	n	% hemocytes showing phagocytyosis	No. particles hemocyte^{-1} showing phagocytosis
Pseudomonas perolens	5	18.0 ± 8.5[a]	4.3 ± 4.2
Pseudomonas perolens treated with mucin	5	7.6 ± 3.9[a]	2.8 ± 2.0
Pseudomonas perolens treated with capsular material	5	10.8 ± 3.7[a]	2.6 ± 2.0

Table 6. *Homarus americanus*. Effects of mucin and *Aerococcus viridans* (var.) *homari* capsular material on phagocytic capacities of hemocytes from lobsters injected 37 to 42 d earlier with vancomycin followed 24 h later with live *Aerococcus viridans* (var.) *homari* (Rabin's). Further details as in Table 3

Opsonized test particles (avirulent strain of *Aerococcus viridans* (var.) *homari* ATCC 10400)	n	% hemocytes showing phagocytyosis	No. particles hemocyte^{-1} showing phagocytosis
ATCC 10400	5	23.6 ± 7.8[a,*]	5.1 ± 4.8[a]
ATCC 10400 treated with mucin	5	11.2 ± 5.3[b]	3.0 ± 1.5[a]
ATCC 10400 treated with capsular material	5	6.8 ± 4.1[b]	3.1 ± 2.1[a]

DISCUSSION

'A well developed non-specific phagocyte-based defensive capability comprises the first line of defense in shellfish and finfish. Phagocytic cells not only recognize, engulf and destroy microorganisms, but can also be involved in cellular encapsulation, production of immunoregulatory molecules, participation in lymphocyte responses, and the processing and presentation of antigens' (Anderson 1993). This dominant view emphasizes that these defenses are essentially non-specific and, although expandable, lack longevity and act as 'a rapid reaction force' (Smith et al. 2003).

With immunogens prepared from Gram-negative bacteria (killed cells or LPS) the induction is non-specific and appears to be dependent on immunogen concentration, but not on its source (e.g. McKay & Jenkin 1969, 1970a,b, Paterson et al. 1976, Paterson & Stewart 1979). The results of the present study using the LPS of *Pseudomonas perolens* are consistent with the earlier findings, as the LPS was non-specific and merely significantly increased the innate phagocytic values for all test particles with the exception of those for the virulent Gram-positive *Aerococcus viridans* (var.) *homari*, strain. Interestingly, although McKay & Jenkin (1969, 1970a,b) were able to induce increased resistance and phagocytic values routinely with cells and LPS from an array of Gram-negative organisms, their attempts to repeat these results with killed Gram-positive cells were unsuccessful.

High degrees of resistance to infection and significant increases in specific phagocytic responses, however, have been induced using Gram-positive cells, i.e. modified *Aerococcus viridans* (var.) *homari*, as the immunogen. The modifications included (1) the vancomycin/live pathogen combination (Stewart & Zwicker 1974), (2) Rittenburg's (1981) culture of the virulent strain of the pathogen in medium containing ca. 2 µg of vancomycin ml^{-1} (i.e. a growth-inhibiting, but not a killing level) followed usually by formolization, and (3) the application of heat (steam-sterilization) to produce a bacterin from the pathogen (Stewart et al. 2004a). The Rittenburg procedure prevented the formation of the capsule, while heat treatment (steam-sterilization) strips the capsular material from the pathogen (Hermansson et al. 1990, Stewart et al. 2004a). Both procedures increased the permeability of the bacterial cell which could then be agglutinated by lobster hemolymph serum. We suggest that the heightened effectiveness of the vancomycin/live pathogen combination is a result of similar alterations to the pathogen occurring *in vivo*. Injection of the antibiotic 24 h prior to injection of the lobster with a substantial suspension of the live pathogen allows the antibiotic to reach an equilibrium within the live lobster (i.e. approximately equivalent to the concentration [ca. 2 µg ml^{-1}] used *in vitro* by Rittenburg 1981) before the pathogen is introduced. Thus, the introduction of *A. viridans* (var.) *homari* in the presence of a widely dispersed growth-inhibiting, but not killing dose of the antibiotic would be expected to give rise to a live bacterium with reduced virulence, i.e. deprived of its protective capsule, and exhibiting increased cell permeability and restricted growth—in effect, an attenuated strain of the pathogen. As shown by Keith et al. (1992), simply mixing vancomycin with killed cells of the pathogen does not produce an immunogen effective in inducing resistance to challenge with *A. viridans* (var.) *homari.*

The live immunogen produced *in vivo* is much more effective than bacterins composed of dead bacterial cells, and results in substantially higher levels of

resistance to the pathogen (Stewart & Zwicker 1974) — up to 1000-fold greater than observed for the bacterins described by Rittenburg (1981) or Stewart et al. (2004a). In addition, these much higher levels of resistance and phagocytosis were produced by suspensions of the attenuated pathogen consisting of 30 to 50-fold fewer cells in the initial injection (1×10^6 or less cells kg^{-1} BW).

Induction of resistance with killed cells is concentration-dependent (Keith et al. 1992, Stewart et al. 2004a), and presumably results from a specific immunogen(s) present in the bacterial cells in relatively low, but fixed concentrations. In contrast, it appears that the lower numbers of attenuated cells can produce the required immunogen(s) levels *in situ* and maintain these for sufficiently long periods, thus inducing much greater levels of resistance and phagocytosis. A partial explanation could be that by introducing fewer cells, the lobster's system is spared the burden of disposing of the large masses of dead cells present in the killed bacterial suspensions. As a result, more of the appropriate hemocytes would be expected to be available for processing and presentation of the immunogen(s), thereby enhancing induction.

As shown by Paterson & Stewart (1979), a significant increase in phagocytic levels was detectable 8 d after exposure to the *Pseudomonas perolens* LPS, and persisted at the maximum level up to at least Day 80 of their study. These authors suggested that the long latent period for induction of increased phagocytosis and its extended duration indicate that the increase could have resulted from production of new phagocytes possessing new or increased capacities; this same reasoning also applies to the increased resistance to the pathogen, which coincidentally requires the same length of time for induction (Stewart et al. 2004a). If this speculation is correct, it would indicate that the duration of enhanced phagocytosis and resistance is probably a function of the turnover rate for circulating hemocytes, and possibly also for fixed cells.

On the basis of available evidence on hemocytes, Arala-Chaves & Sequeira (2000) argued that it is 'reasonable to assume that a peculiar form of adaptive response, quantitatively and qualitatively different from that of vertebrates probably exists in invertebrates'. Obviously much more work is required to delineate the exact course of events occurring within the lobster. It is evident, however, from the results reported herein, that a highly significant degree of specificity can be induced in the lobster by the use of a particular immunogen. This flexibility in response raises the possibility that at least 1 crustacean genus is capable of adaptive responses and that homarid lobsters and the disease gaffkemia constitute a good model system for exploring these fundamental responses further.

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